INTRODUCTION
Chondrocytes from the resting zone of the growth plate are regulated by the vitamin D metabolite 24,25-dihydroxyvitamin D3 (24R,25(OH)₂D₃), with increased differentiation and the production of extracellular matrix and reduced matrix metalloproteinase activity. The effect of 24R,25(OH)₂D₃ is mediated by the activation phospholipase D (PLD(1)). PLD activation results in increased production of lysophosphatidic acid (LPA), a bioactive lysophospholipid that promotes cell proliferation, survival, apoptosis, and migration. Recent studies have demonstrated a role for LPA in cartilage and bone formation, suggesting that LPA is a second messenger during chondrocyte maturation and survival in growth plate cartilage cells. To test this hypothesis, we used resting zone chondrocytes isolated from rat growth plate as our model system. We examined the effect of 24R,25(OH)₂D₃ on LPA synthesis and LPA receptor expression. Furthermore, we examined if 24R,25(OH)₂D₃-mediated chondrocyte maturation and cell survival are attenuated with LPA receptor antagonist.

Clinical Significance: Information gleaned from this study will lead to a better understanding of the mechanisms of vitamin D metabolite signaling in the growth plate for the development of novel therapies for fracture repair and growth plate disorders.

METHODS

Cell Culture. Chondrocytes were obtained from the resting zone (reserve zone) of costochondral cartilage from 125-g male Sprague-Dawley rats and cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% antibiotics, and 50 µg/ml ascorbic acid. Chondrocyte Maturation Assays. Alkaline Phosphatase Activity: Cell monolayers were harvested from resting zone chondrocytes using 0.1% Triton X followed by sonication of each cell layer lysate as a function of release of para-nitrophenol from para-nitrophenylphosphate at pH 10.2. Sulfate Incorporation: Cells were labeled with [³⁵S]-sulfate 3 h prior to harvest. At harvest, the conditioned media was removed, the cell layers were collected, and the amount of [³⁵S]-sulfate incorporated was determined as a function of protein in the cell layer. Expression of LPA receptors-Real-Time PCR. cDNA was generated via RT-PCR and quantitative real-time PCR was conducted using sequence specific primers for each of the LPA receptors. Production of LPA-LC ESI MS/MS: The abundance of LPA isoforms in lysates and media collected from the resting zone chondrocytes was determined by liquid chromatography electrospray ionization tandem mass spectrometry (LC ESI MS/MS). Cells were treated for 30 minutes with starving media (1% FBS) containing 1% bovine serum albumin. After the treatment period, 1ml of conditioned media was collected and cell monolayers were harvested using 0.05M NaOH. Media and lysate samples were spiked with lpmol of 17.0 LPA (internal standard) prior to lipid extraction via the Bligh-Dyer method. LC ESI MS/MS analysis was conducted in the Georgia Institute of Technology Mass Spectrometry Center using a Shimadzu HPLC pump and a Q-TRAP 4000 (Applied Biosystems). Measure of Apoptosis-Caspase-3 Activity. Resting zone chondrocytes were treated with 7.5mM monobasic sodium phosphate (Pi) in the presence or absence of 24R,25(OH)₂D₃. Apoptosis was assessed by measuring caspase-3 activity (Promega) normalized to total protein.

RESULTS
24R,25(OH)₂D₃ increases the abundance of extracellular LPA in a dose dependent manner (Figure 1A). Real-Time PCR showed that RC cells expressed all known LPA receptors (LPA1-5 and PPAR-γ). Only LPA1 mRNA expression was increased by 24R,25(OH)₂D₃ (Figure 1B). Taken together, these results demonstrate that 24R,25(OH)₂D₃ is a regulator of LPA signaling. Based on these findings, we hypothesized that LPA was a second messenger in the 24R,25(OH)₂D₃ signaling pathway. Both 24R,25(OH)₂D₃-mediated increases in alkaline phosphatase and proteoglycan sulfation were abolished by the LPA1/3 selective antagonist VPC32183(S) in a dose dependant manner. Moreover, VPC32183(S) also inhibited the rescue of Pi-mediated apoptosis by 24R,25(OH)₂D₃. These data show that LPA is a necessary down-stream component of the 24R,25(OH)₂D₃-mediated chondrocyte maturation and cell survival.

DISCUSSION
24R,25(OH)₂D₃ increases both the production of LPA and the expression of LPA1 establishing a role for the vitamin D metabolite as a regulator of LPA signaling in resting zone chondrocytes. Furthermore, 24R,25(OH)₂D₃-mediated maturation and cell survival are attenuated by LPA1/3 selective antagonist VPC32183(S). Collectively these data confirm that LPA is a second messenger in the 24R,25(OH)₂D₃-mediated chondrogenesis. This establishes a novel physiological function of LPA signaling and provides insight into the mechanism behind the action of vitamin D metabolites in the growth plate.

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